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(54) Title: IL-5R ANTAGONISTS FOR TREATMENT OF INFLAMMATION, ASTHMA AND OTHER ALLERGIC DISEASES

(57) Abstract

Disclosed are antagonist molecules, including monoclonal antibodies, which bind to the extracellular portion of the α chain of the IL-5 receptor protein, and inhibit proliferation of eosinophils as measured in an *in vitro* assay. Two such monoclonal antibodies which were particularly effective in such binding were found to bind, respectively, to the portion of the sequence of human IL-5 receptor shown in SEQ ID NO:2, and to a conformational epitope.

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IL-5R Antagonists for Treatment of Inflammation, Asthma and Other Allergic Diseases

FIELD OF THE INVENTION

The invention relates to antagonists for the interleukin-5 receptor for treating inflammation and asthma and other allergic diseases.

BACKGROUND OF THE INVENTION

The pathology of asthma is associated with infiltration of eosinophils into the bronchoalveolar tissue. *In vitro* studies have shown that the accumulation of eosinophils is associated with the presence of TH2 cells and mast cells and the production of interleukin-5 ("IL-5") (Huston, D.P., Postgraduate Syllabus, pp. 127-142 (1997)). Animal studies further confirmed that IL-5 plays a key role in the asthmatic response. A neutralizing anti-IL-5 antibody has been shown to inhibit antigen or virus induced airway hyper-responsiveness and eosinophil inflammation in the airways of mice (Kung, TT., American J. Respiratory Cell & Molecular Biology, 1995, 13:360-5).

Eosinophils have a receptor protein for IL-5 on their surface. The interaction between this receptor and IL-5 may be associated the inflammatory response, and particularly, with asthma. Preventing this interaction could therefore play an important role in reducing inflammation and/or asthma. There has been one suggestion of use of an antagonist

EP 0811691 A1. The molecules of the present invention are believed to be superior to those disclosed therein.

SUMMARY OF THE INVENTION

The invention includes antagonists of the IL-5 receptor ("IL-5R"), for example, antibodies and fragments, homologues and analogues thereof, as well as peptides, nucleotides, or other antagonist molecules, including immunoglobulin fragments like Fab, $(Fab')_2$ and Fv, and small molecules, including peptides, oligonucleotides, peptidomimetics and organic compounds. These receptor antagonists can bind to the IL-5R on the eosinophils or eosinophil progenitor cells but do not stimulate them. Therefore, they compete with IL-5 for receptor sites, and inhibit the functional activity of IL-5. This in turn inhibits the pathogenesis of inflammation, asthma and the airway allergic response. These antagonists can all be screened using methods that are the same or essentially the same (with some modification) as those described below, which were used to isolate certain antibodies which are IL-5R antagonists.

The preferred anti-IL-5R monoclonal antibodies of the invention are chimeric, humanized, or human antibodies, or other antibody derivatives which are less immunogenic than antibodies which are wholly non-human, such as murine antibodies. One preferred monoclonal antibody of the invention binds to an epitope on human IL-5R having the sequence set forth in SEQ ID NO: 1. Another binds to a conformational epitope on the

human IL-5R. Other preferred antagonist molecules would be expected to bind to the same epitopes. However, the screening methods described below can also be used to isolate antibodies or other antagonist molecules binding to other epitopes, some of which may be superior in their antagonist effect to those targeted by the antibodies 165-4 and 165-13 described below.

The antibodies and other antagonist molecules of the invention can be used therapeutically or diagnostically. In diagnostic applications, they can be used to detect or quantify eosinophils in a body fluid sample, using conventional analytical techniques.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1 depicts the specific binding of three different antagonist monoclonal antibodies to human IL-5 receptor alpha chain as determined by ELISA. An isotype matched monoclonal antibody was set as a control in this assay.

Figure 2 depicts blocking of human IL-5 binding to IL-5 receptor alpha chain by three different anti-IL-5Rα antagonist monoclonal antibodies, as determined by ELISA. The decrease in $OD_{450/570}$ readings indicates the decrease of IL-5Rα/IgG4Fc binding to human IL-5, resulting from competition of the antagonist monoclonal antibodies binding to human IL-5 receptor alpha chain.

Figure 3 depicts competition [illegible] three different antagonist

monoclonal antibodies binding to free IL-5Rα/IgG4Fc with the antagonist monoclonal antibody 165-13, as determined by ELISA.

Figure 4 depicts proliferation inhibition of the IL-5 dependent human cell line TF-1 by two different human IL-5Rα antagonist antibodies in the presence of rhIL-5 (A), or rmIL-5 (B) with a final concentration of 0.3 ng/ml, by MTT assay. The irrelevant isotype matched control monoclonal antibody 165-21 had no effect.

Figure 5 depicts comparison of proliferation inhibition effects of soluble human IL-5 receptor alpha chain and human IL-5Rα antagonist monoclonal antibody 165-13 in the presence of soluble hIL-5 with a final concentration of 0.3 ng/ml by MTT assay.

Figure 6 depicts the results of the eosinophil adherence assays. hIL-5 stimulated eosinophils adhere to human IgG coated wells in a dose dependent manner. When different concentrations of the anti-IL-5 monoclonal antibody 165-13 was added to the eosinophil cultures, the stimulatory effect of hIL-5 was inhibited in a dose-dependent manner, whereas the irrelevant isotype matched control monoclonal antibody had no effect. □ represents IL-5 alone; ▲ represents IL-5 and the 165-13 mAb at 10μg/ml; ◆ represents IL-5 and the 165-13 mAb at 2μg/ml; ● represents IL-5 and the 165-13 mAb at 0.4μg/ml; O is an isotype control antibody; and ▽depicts no IL-5 added.

MAKING AND USING THE INVENTION

The IL-5R consists of an IL-5-binding α chain and a non-binding β chain, which is shared with the IL-3 receptor and the GM-CSF receptor. Therefore, preferred antagonists should target the α chain rather than the β chain. Such molecules can be of a number of different types, including antibodies, fragments, homologues and analogues thereof, as well as peptides, oligonucleotides, peptidomimetics, organic compounds and other small molecules targeting the α chain and acting as antagonists. All these antagonists can screened by methods similar to those used to screen for the preferred antibodies, as described below.

In one embodiment, the invention relates to anti-IL-5R α chain antagonist monoclonal antibodies. Monoclonal antibodies can be produced by immunization and fusion, or production from isolated lymphocytes and EBV transformation. If one is producing them through immunization, the anti-IL-5R α chain peptide, or a fragment or derivative thereof, is preferred for the immunogen, and more preferably, the portion of this peptide which lacks a substantial hydrophobic region.

The anti-IL-5Rα chain peptide is a single chain peptide. One of the methods to make such peptides more immunogenic and more suitable for antibody generation is to produce an Fc fusion protein, which includes this peptide fused with a Fc portion of an immunoglobulin, optionally with a

immunogenicity of the chimeric protein, but also simplifies purification of the immunogen when using affinity chromatography. The specific method of making and purifying this fusion protein is described in Examples 2 to 5. It should be understood that other immunogens can also be used, including portions or fragments of the IL-5R α chain, and, preferrably, any extracellular portion which is exposed and accessible for antibody binding.

The immunogens so generated can be used to make polyclonal or monoclonal antibodies, or fragments thereof, or can be used to screen libraries (including phage display libraries and peptide or compound libraries) for molecules which bind thereto. Such methods of producing antibodies and fragments, and of screening, are well known in the art. The molecules selected by these screening methods can then be further characterized for antagonist activity, as described below.

More specifically, the antagonist antibodies of the invention can be generated by immunizing rodents (*e.g.* mice, rats, hamsters or guinea pigs) with the IL-5R immunogen or the fusion protein described above. Other animals can also be immunized, *e.g.* non-human primates, which would produce less immunogenic antibodies. Hybridomas can be generated by conventional procedures by fusing B lymphocytes from the immunized animals with myeloma cells (e.g. Sp2/0 and NS0), as described by G. Köhler and C. Milstein (*Nature*, 1975: 256: 495-497). In addition, antibody fragments can be generated by screening of

recombinant single-chain Fv or Fab libraries from human B lymphocytes in phage-display systems. Although the methods described in Examples 6 to 11 below were used to make the antibodies of the invention, any of the foregoing methods could be used to make such antibodies. These antibodies, or fragments, homologues or analogues thereof, can then be characterized for their antagonist effect, as described in the examples.

The epitope to which the antagonist molecules bind can be determined by the method described in Example 12, or by other methods of epitope mapping, which are well known in the art. The epitope to which the molecules bind may be linear or conformational. The molecules which bind to such epitopes, including antibodies, fragments, homologues and analogues thereof, are described below and included within the invention.

The *in vivo* biofunctional activity of antagonist molecules can be determined by animal studies. The molecules could be applied to the animal's lung surface before contacting the lungs with an allergen. The eosinophil infiltration into the lungs could then be measured and compared with a control to determine the effectiveness of the molecule in asthma or allergic disease treatment.

For administration to humans, the antibodies of the invention would preferably be administered as chimeric, humanized or human antibodies. Such antibodies can reduce immunogenicity and thus avoid or minimize a

preferable that the antibody administered be IgG_4, IgG_2, or other genetically mutated IgG or IgM which does not augment antibody-dependent cellular cytotoxicity (S.M. Canfield and S.L. Morrison, *J. Exp. Med.*, 1991: 173: 1483-1491) and complement mediated cytolysis (Y.Xu et al., *J. Biol. Chem.*, 1994; 269: 3468-3474; V.L. Pulito et al., *J. Immunol.*, 1996; 156: 2840-2850).

Chimeric antibodies are produced by recombinant processes well known in the art, and have an animal variable region and a human constant region. Humanized antibodies have a greater degree of human characteristics than do chimeric antibodies. In a humanized antibody, only the complementarity determining regions (CDRs), which are responsible for antigen binding and specificity, are animal derived and have an amino acid sequence corresponding to the animal antibody. Substantially all of the remaining portions of the molecule (except, in some cases, small portions of the framework regions within the variable region) are human derived and correspond in amino acid sequence to a human antibody. See L. Riechmann et al., *Nature*, 1988; 332: 323-327; G. Winter, *United States Patent* No. 5,225,539; C.Queen et al., *United States Patent No. 5,585,089*.

Human antibodies can be made by several different ways, including immunization and fusion of cells obtained from transgenic mice expressing human immunoglobulins, or from severe combined

immunodeficient (SCID) mice transplanted with human B lymphocytes, which are then used to produce hybridomas producing the human antibodies. Transgenic mice are available from Abgenix, Inc., Fremont, California, and Medarex, Inc., Annandale, New Jersey. Alternatively, one can use human immunoglobulin expression libraries (Stratagene Corp., La Jolla, California) to produce fragments of human antibodies (V_H, V_L, Fv, Fd, Fab, or $(Fab')_2$, and use these fragments to construct whole human antibodies using techniques similar to those for producing chimeric antibodies. Single peptide chain binding molecules in which the heavy and light chain Fv regions are connected can be made by known methods. (A. Plückhun. *Methods: a companion to Meth. Enzymol.*, 1991: 2: 88-96; M. Whitlow and D. Filpula, *ibid*, 97-105), as can Fab fragments (M.J. Evans et al., J. Immunol. Meth., 1995; 184: 123-138), and other fragments. All of the wholly and partially human antibodies and fragments are less immunogenic than wholly animal or murine monoclonal antibodies. All such molecules are therefore less likely to evoke an immune or allergic response and therefore are better suited for *in vivo* administration in humans than animal or murine antibodies, especially when repeated or long-term administration is necessary.

Based on the molecular structures of the variable regions of the antibodies of the invention, one could use molecular modeling and rational

molecular structures of the binding region of the antibodies and are also effective IL-5R antagonists. These small molecules can be peptides, peptidomimetics, oligonucleotides, or other organic compounds. Alternatively, one can screen libraries of such molecules by the methods set forth in the examples below. All such methods are known to those skilled in the art, and would yield small molecules which are effective antagonists.

Example 1. Eosinophil isolation:

Fifty milliliters of buffy coat unit (prepared from 450 ml blood, Houston Blood Center, Houston, TX) were diluted with equal volume of buffer A (13.8 mM Na3 citrate-$2H_2O$, 1% BSA in PBS, pH 7.0). Forty microliters of diluted blood were overlaid onto 50 ml conical tubes containing 10 ml of Ficoll-Paque, and then centrifuged at 18°C - 20°C at 400 g (IEC, 1300rpm) for 30 minutes. After collection of granulocytes (including contaminating erythrocytes) from the bottom of the tubes, the erythrocytes were lysed with 5 x volume of an ice-cold isotonic ammonium-chloride solution (0.83% NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA) at 0°C for 15 minutes. After centrifugation for 7 minutes at 400g and 4°C, the granulocytes were washed with 1 x PBS plus 1% BSA, suspended in the same solution and then incubated for 30 minutes at 37°C. The cells were washed with 1 x buffer A, and resuspended at a concentration of 7.5×10^7 cells/ml in buffer A. After incubation of the cells for 5 minutes at 37°C, 1 μl of FMLP (10 μM in PBS) was added and

incubated for 10 minutes. One ml of cell suspension was laid onto a 2-layer Percoll gradient in which 1 ml of Percoll (density: 1.100 g/ml) was inserted in the bottom and another 4 ml of Percoll (density: 1.082 g/ml) on the top in a 15 ml conical tube. The eosinophils from the interface between the two Percoll layers were collected, after centrifugation at 1000g for 15 minutes at room temperature.

Example 2. Cloning of the cDNA segment encoding human IL-5 receptor alpha chain.

Total RNA was prepared by the Ultraspec-3 RNA isolation kit from about 10 x 10^6 eosinophil cells obtained as above, according to the instructions of the manufacturer (Biotecx Laboratories Inc., Houston, TX). The single strand cDNA for human IL-5 receptor alpha chain was synthesized by a reverse transcription reaction in which about 5 μg of total RNA obtained as above were added into 20 μl of reaction mixture, according the protocol of the manufacturer (Gibco BRL, Gaithersburg, MD). 2 μl of this reverse transcription reaction mixture was added into 100 μl of PCR reaction mixture which contains two primers (97320-1: TAA AGC TTG ACA GGA TAT GAT CAT CGT (SEQ ID NO:3); 97320-3: TCG GAT CCC ACA TAA ATA GGT T (SEQ ID NO:4)) at a final concentration of 200 nM. PCR reaction was carried out in the Geneamp 9600 (Perkin Elmer). After precipitation, the PCR product was digested with BamH1 and HindIII, and ligated into the cloning vector pUC18, which was also digested with BamH1 and HindIII, yielding the recombinant plasmid DNA

Both strands of the insert in the plasmid pN10 were confirmed by DNA sequencing.

Example 3. Construction of the mammalian expression plasmid for human IL-5Rα/IgG4Fc chimeric protein:

The DNA fragment encoding the extracellular portion of human IL-5Rα chain was isolated from the plasmid pN10 by digestion with HindIII and BamH1, and inserted into the plasmid pcDNA3Fc, which contains an inserted DNA fragment encoding human IgG4Fc and a short DNA fragment encoding a peptide linker, yielding the mammalian expression plasmid pN3. In this expression plasmid pN3 the expression of the chimeric protein, consisting of the extracellular portion of the human IL-5Rα chain in-frame fused with the linker and IgG4Fc, is under the control of hCMV promoter. After linearization with BspC1, the plasmid pN3 was electrotransfected into NSO cells, using a BioRad apparatus.

Example 4. Expression of human IL-5Rα/IgG4Fc in mammalian cells:

NSO cells were transfected with linearized pN3. 4 $X10^7$ log-phase NSO cells was harvested and resuspended in 0.8 ml IMDM medium supplemented with 2% FBS. After incubation with 10 μg of linearized plasmid DNA for 10 minutes on ice, the cell mixture was subjected to electroporation at 200 volts and 960 μF, using a BioRad apparatus. After keeping it on ice for 20 minutes, 100 μl of cell suspension was added to each well of about twenty 96-well plates. Two days later, another 100 μl of the same IMDM medium but containing G418 (Gibco BRL, Gaithersburg,

MD) was added into each wells to make the final concentration of G418 0.8 mg/ml. After 10 days, culture supernatants were withdrawn.

Screening for the expression of the extracellular portion of human IL-5 receptor alpha chain/IgG4Fc fusion protein was done by ELISA as follows. The wells of Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) were precoated by adding 50 μl of goat anti-human IgG(Fc) antibody at concentration of 1 μg/ml and stored overnight at room temperature. After the coating solution was removed by flicking the plates, 200 μl of BLOTTO (5% non-fat dry milk in PBST) was added to each well at room temperature to block non-specific bindings. One hour later, wells were washed with PBST buffer (PBS containing 0.05% Tween 20). Fifty microliters of culture supernatants from each well in the transfection plates were collected and mixed with 50 μl of BLOTTO, and then added to individual wells of the microplates. After one hour of incubation at room temperature, the wells were washed with PBST. The bound extracellular human IL-5 receptor alpha chain/IgG4Fc fusion protein was detected by reaction with horseradish peroxidase-conjugated goat anti-human IgG(Fc) (Jackson ImmunoResearch Laboratories, West Grove, PA), which was diluted at 1:2000 in BLOTTO. Peroxidase substrate solution containing 0.1% 3,3,5,5 tetramethyl benzidine (Sigma, St. Louis, MO) and 0.0003% hydrogen peroxide (Sigma) was added to each well for color

50 μl of 0.2 M H_2SO_4 per well. The $OD_{450-570}$ reading of the reaction mixture was measured with a BioTek ELISA Reader (BioTek Instruments, Winooski, VT).

The transfectants with high $OD_{450-570}$ reading were picked up and single cell cloning was performed by the limiting dilution method. The ELISA as above was repeated to more specifically identify the high producer cell lines expressing the fusion protein of the extracellular portion of the human IL-5 receptor alpha chain/IgG4Fc chimeric protein.

Example 5. Purification of the extracellular portion of human IL-5 receptor alpha chain/IgG4Fc chimeric protein:

Two liters of the culture supernatant from the transfectant cells expressing the extracellular portion of human IL-5 receptor alpha chain/IgG4Fc chimeric protein was collected, and this chimeric protein was purified by Prosep-A affinity chromatography, according to the manufacturer's instruction (Bioprocessing Inc., Princeton, NJ). The purity of this purified chimeric protein was determined by both SDS-PAGE and immunobloting.

Example 6. Hybridoma generation:

Five BALB/c mice, 8 weeks old, were injected subcutaneously with 30 μg of purified the extracellular portion of human IL-5 receptor alpha chain/IgG4Fc chimeric protein emulsified with complete Freund's adjuvant (Difico Laboratories, Detroit, MI) in 200 μl of phosphate buffered saline (PBS), pH 7.4. The mice were boosted after 2 and 4 weeks with the same

antigen in incomplete Freund's adjuvant. Two weeks later and three days prior to sacrifice, the mice were given a final booster injection i.p. and their spleen cells were fused with Sp2/0 myeloma cells. 5 x 10^8 of the Sp2/0 cells and an equal number of spleen cells were fused in a medium containing 50% polyethylene glycol (MW 1450)(Kodak, Rochester, NY) and 5% dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO). The cells were adjusted to a concentration of 5 x 10^4 spleen cells per 200 μl of the suspension in Iscove medium (Gibco BRL, Gaithersburg, MD), supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of Streptomycin, 0.1 mM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine. Two hundred microliters of the cell suspension was added to each well of one hundred 96-well microculture plates. After about ten days culture supernatants were withdrawn to screen for the antibodies' ability to compete for binding to the human IL-5 receptor alpha chain/IgG4Fc chimeric protein with human IL-5 protein. The competitive hybridoma colonies from these supernatants were further subcloned and expanded.

Example 7. Competitive ELISA:

For screening antagonist antibodies against human IL-5Rα from the hybridoma supernatants, one hundred Immulon 2 microtiter plates were precoated with rat anti-human IL-5 monoclonal antibody JES1 (R&D Biosystems) at the final concentration of 1 [illegible] in PBS [illegible] temperature. Fifty [illegible] containing [illegible]

concentration of 0.06 μg/ml was placed in each well and kept at room temperature for one hour. After washing with PBST, 100 μl of mixture containing 50 μl of the hybridoma supernatants and 50 μl of human IL-5Rα/IgG4Fc chimeric protein at a final concentration of 0.5 μg/ml was added to each well in these plates, and maintained for one hour at room temperature. After washing with PBST, 50 μl of goat anti-human IgG(Fc) conjugated with horseradish peroxidase (diluted 1:5000) was added to each well, and incubated for one hour at room temperature. After washing with PBST, peroxidase substrate solution containing 0.1% 3,3,5,5 tetramethyl benzidine (Sigma, St. Louis, MO) and 0.0003% hydrogen peroxide (Sigma) was added to each well for color development. The reaction was terminated after 30 minutes by adding of 50 μl of 0.2 M H_2SO_4 per well. The $OD_{450-570}$ reading of the reaction mixture was measured with a BioTek ELISA Reader (BioTek Instruments, Winooski, Vermont). Lower $OD_{450-570}$ readings of the wells indicated stronger inhibition ability of the supernatant to interaction of IL-5 with the IL-5 receptor alpha chain.

To test binding abilities of these purified antagonist monoclonal antibodies obtained as in Example 8 below, the following procedure was performed. 50 μl of goat anti-mouse antibody IgG(Fc) in BLOTTO at a final concentration of 0.5 μg/ml per well was precoated at room temperature overnight. After the coating solution was removed by

flicking the plate, various amounts of purified antagonist monoclonal antibodies in BLOTTO was added to each well and the plate was incubated at room temperature for one hour. Then the plate was washed with PBST, and 50 μl of human ("h")IL-5Rα/IgG4Fc chimeric protein at a final concentration of 0.5 μg/ml was added to each well, continued at room temperature for one hour, and then washed with PBST. The plate was coated with HRP-conjugated goat anti-human IgG(Fc) for one hour and then peroxidase substrate solution was added to each well. The results for three different antibodies are shown in Fig. 1.

To determine the competition ability of the purified antagonist monoclonal antibodies obtained as above with human IL-5Rα/IgG4Fc, an Immulon 2 plate was coated with 50 μl/well (0.5 μg/ml) of goat anti-mouse monoclonal antibody IgG(Fc) in PBS. Then the plate was washed with PBST and coated with 50 μl/well (0.5 μg/ml) of human IL-5Rα antagonist monoclonal antibody 165-13 at room temperature for one hour. After washing with PBST, 100μl of BLOTTO containing the mixture of hIL-5Rα/IgG4Fc (0.5 μg/ml) and various amounts of antagonist antibodies obtained as above were added to each well, and incubated at room temperature for one hour. The plate was washed with PBST and HRP-conjugated goat anti-human IgG(Fc) was added. The results for three different antibodies are shown in Fig. 2.

monoclonal antibodies obtained in Example 8, a similar competitive ELISA as described above for screening antagonist antibodies was performed, except that after coating with human IL-5 at room temperature for one hour and washing with PBST, various amount of purified monoclonal antibodies with hIL-5Rα/IgG4(Fc) at a final concentration of 0.5 μg/ml was added to each well. After incubation at room temperature for one hour and washing with PBST, the same steps were conducted as described above for the competitive ELISA for screening the antagonist antibodies in hybridoma supernatants. The results for three different antibodies are shown in Fig. 3.

Example 8. Purification of anti-human IL-5 receptor alpha chain antagonist monoclonal antibodies:

The monoclonal antibodies generated from hybridomas described in Example 7 were purified by Prosep-A affinity chromatography, according to the manufacturer's instruction (Bioprocessing Inc., Princeton, NJ). The purity of these monoclonal antibodies was checked by SDS-PAGE and Western blot.

Example 9. Proliferation inhibition assay of IL-5 dependent cells in the presence of hIL-5 by IL-5R antagonist monoclonal antibodies:

TF-1 maintained in hIL-5 containing medium (RPMI-1640 + 10% FBS + 5 ng/ml hIL-5) was washed with hIL-5 free medium three time, and resuspended in a hIL-5 (Fig. 4(A)) or mouse ("m") IL-5 (Fig. 4(B)) containing medium (RPMI-1640 + 10% FBS + 0.6 ng/ml hIL-5 or mIL-5,

respectively) at a final cell density of 2 x 10^4/100 μl. Fifty microliters of TF-1 cells were added to each well of a 96-well plate. Fifty microliters of varying amounts of purified IL-5Rα antagonist monoclonal antibodies obtained as above was added to each well. After three days of incubation, 10 μl of MTT (2.5 mg/ml in PBS) was added to each well and incubated for 4 hours. One hundred μl of solubilization solution containing 10% SDS and 0.01 N HCl was added and the plate was incubated overnight. The proliferation of these IL-5 dependent TF-1 cells was monitored by reading at $OD_{540-690}$.

Example 10. Competitive inhibition assay of IL-5 dependent cells in the presence of hIL-5 by soluble human IL-5Rα protein or antagonist monoclonal antibodies:

TF-1 cells maintained in hIL-5 containing medium (RPMI-1640 + 10% FBS + 5 ng/ml hIL-5) were washed with IL-5-free medium three times and resuspended in hIL-5 containing medium (RPMI1640 + 10% FBS + 0.6 ng/ml hIL-5) at a final cell density of 2 x 10^4/100 µl. Fifty microliters of the cells was added to each well of a 96-well plate. Fifty microliter with varying amounts of purified hIL-5 alpha chain (R & D Biosystems) was added to each well. After three days of incubation, 10 μl of MTT (2.5 mg/ml in PBS) was added to each well and incubation was continued for 4 hours. One hundred microliters of solubilization solution as described in Example 9 was added and the plate was incubated overnight. The proliferation of these IL-5 dependent TF-1 cells

at $OD_{540-690}$ The results for the antibody 165-13 against the hIL-5Rα chain are shown in Fig. 5.

Example 11. Adherence assay of IL-5 activated human eosinophils to a plastic culture plate immobilized with human IgG:

Eosinophils were purified from a "buffy coat" collected from a healthy blood donor. The buffy coat was mixed with 3% Dextran T-500 (in 0.9% NaCl) at a 1:1 (v/v) ratio. The mixture was allowed to settle for 20 minutes until a clearly defined interphase appeared. The upper layer was collected and centrifuged at 300 x g for 10 minutes at 4°C. The cell pellet was resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. The cell suspension was layered on 10 ml of Ficoll-Paque in a 50-ml tissue culture tube. The tube was centrifuged at 400xg for 40 minutes at 20°C. At the end of the centrifugation, the cell pellet which contains polymorphonuclear cells and red blood cells was collected. The red blood cells were then lysed using 0.83% ammonium chloride solution for 10 minutes at 4°C. The cell suspension was centrifuged and washed, and resuspended in 25 ml of 0.2% cold sodium chloride solution. 30 seconds later, 25 ml of 1.6% cold sodium chloride solution was added. The cell suspension was then centrifuged at 300 x g for 6 minutes at 4°C. The cell pellet was then resuspended in PBS and loaded on a Percoll gradients (density: 1.085, 1.090, 1.095 and 1.100 g/ml) in a tissue culture tube. The tube was centrifuged at 700 x g for 20 minutes. At the end of the centrifugation, cells at the interphase of the

gradient 1.095/1.100 g/ml were collected. The cells contained mainly eosinophils.

For the adherence experiments, purified human IgG in PBS was added (1 μg/well) to each well of 96-well tissue culture plates and incubated for 2 hours at 37°C. The plates were washed 3 times with PBS. The plates were then blocked with 50% (v/v) fetal bovine serum-PBS for 2 hours at 37°C. The plates were washed with adhesion medium (Hanks balanced salt solution with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% human serum albumin) to remove the blocking buffer. To each well, 50 μl of either adhesion medium or recombinant IL-5 (100 pM) was added with 50 μl of eosinophil suspension (10^5 cells/ml in adhesion medium). One hundred microliters of anti-IL-5 monoclonal antibody 165-13 (produced, purified and screened as in the examples above) of different concentration were added in duplicate to the test wells. For negative control, an irrelevant isotype control monoclonal antibody was used. The plates were incubated for 30 minutes at 37°C, and then washed three times with the adhesion medium. The adherent eosinophils were lysed with 50 μl of 0.3% (w/v) cetyl trimethylammonium bromide, followed by addition of 100 μl of eosinophil peroxidase substrate (o-phenylenediamine). After 30 minutes at room temperature, the color reaction was terminated by addition of 50 μl of 2 M H_2SO_4 and the OD_{490} nm read, after blanking on wells containing eosinophils

direct measure of eosinophil peroxidase and was used as an indirect measure of the number of eosinophils adhered to each well and the binding ability of the antibody 165-13 to eosinophils. The results are shown in Fig. 6.

Example 12: Epitope mapping of Anti-IL5 receptor antibodies

A. Constructing the human interleukin-5 receptor alpha chain epitope library.

Seven micrograms of pcDNA3 plasmid containing the sequence coding for the human interleukin-5 receptor alpha chain (pcDNA3-IL-5Rα) was used to determine the conditions for DNase digestion which will yield 50-100 bp of DNA fragments. Aliquots of the plasmid were dissolved in DNase I buffer (50 mM Tris, pH7.6, 1 mM MnCl, 0.1 mg/ml BSA) and placed in seven 1.5-ml eppendorf tubes. 3.5 μl of seven different enzyme dilutions (0, 0.312, 0.625, 1.25, 2.5, 5, 10 units/ml) were added to the respective eppendorf tubes containing the plasmid, and all the tubes were incubated at 15°C for 10 minutes. The digestion was stopped by adding 2 μl of 250 mM EDTA. The sample was loaded into each tube onto 2.5% agarose gel (SEAKEM LE agarose, FMC) for analysis. The enzyme dilution that gives the highest yield of 50-100 bp DNA fragments was selected. 20 μg pcDNA3-IL-5Rα was digested by using the selected enzyme digestion conditions. The DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation, and redissolved in 100 μl repair buffer (40 mM Tris-HCl, pH 8.0, 10 mM ammonium sulfate, 10 mM 2-

mercaptoethanol, 5 mM magnesium chloride, 0.5 mM EDTA, 100 μM dNTP). The ends were repaired using 30 U of T4 DNA polymerase (NEB) at 15°C for one hour and 10 U of Klenow fragment (NEB) at 37°C for 30 minutes. The repaired DNA sample was loaded on a 2.5% low-melting agarose gel (SEAPLAQUE agarose, FMC) and 50-100 bp DNA fragments were isolated from the gel. The DNA was purified using gelase.

The concentration of the 50-100 bp DNA fragments was quantitated on 2.5% agarose gel. Using 10 U T4 ligase (BRL, 10U μl), the DNA fragments were ligated at 16°C overnight with a vector containing the blunt ends. The ligated DNA was purified through phenol/chloroform extraction and ethanol precipitation, and transformed into TG1 cells (*supE hsd$_{\Delta}$5 thi $_{\Delta}$(lac-proAB)* F'[*traD36 proAB^{+} lacI^{q} lacZ*ΔM15]) by electroporation. About 2 million individual clones were obtained.

B. Screening for phage particles displaying the epitopes for antibodies.

The phage-display epitope library for IL-5Rα was rescued from a 100-ml culture using the helper phage M13KO7. The resultant phage solution was concentrated using PEG precipitation (13% PEG8000, 0.15M NaCl). The concentrated phage library was panned against two anti-IL5Rα murine antibodies, mAb165-4 (screened in as in Example 10) and mAb165-13, for three rounds (1st round, coat 5 υg/ml mAb, wash 6 x PBST, 4 x PBS; 2nd round, coat 5 μg/ml mAb, wash 10 x PBST, 3 x PBS;

characterization of individual phage clones using ELISA and DNA sequencing analysis.

C. Confirming the binding and analyzing the sequence of individual clones.

The individual clones were selected after three rounds of bio-panning and the phage particles derived from these individual clones were subjected to ELISA analysis. Four clones that bind to mAb 165-4 and five clones that bind to MAb 165-13 were sequenced and the sequencing results were aligned with the sequence of the human IL-5Rα (SEQ ID NO: 1). The consensus sequence of all four mAb 165-4-binding clones was found to be as follows: Ser Ile Gln Trp Glu Lys Pro Val Ser Ala Phe Pro Ile His Cys Phe (SEQ ID NO.: 2, which is underlined in SEQ ID NO.: 1). No consensus sequence for MAb 165-13 was found, indicating that it binds to a conformational epitope. The specific methods of making certain murine antagonist antibodies of the invention are described below. As noted above, the same or similar methods can be used to generate other antibodies or antagonists of the invention.

It should be understood that the examples, terms and expressions used herein are exemplary only and not limiting, and that the invention is defined only in the claims which follow, and includes all equivalents of those claims.

SEQUENCE LISTING

(1) General Information:
(i) Applicant: Yu, Liming; Sun, Bill; Sun,Cecily; Ni; Paul
(ii) Title of Invention: IL-5R Antagonists for Treatment of Inflammation, Asthma and Other Allergic Diseases
(iii) Number of Sequences: 4
(iv) Correspondence Address:
(A) Addressee: Tanox Biosystems, Inc.
(B) Street: 10301 Stella Link Rd.
(C) City: Houston
(D) State: Texas
(E) Country: USA
(F) Zip: 77025
(v) Computer Readable Form:
(A) Medium Type: Diskette, 3.5 inch
(B) Computer: Addonics C142 SVGA
(C) Operating System: Microsoft Windows 95
(D) Software: Word
(vi) Current application data:
(A) Application Number:
(B) Filing Date:
(C) Classification:
(vii) Prior Application Data:
(A) Application Number: 60/044,705
(B) Filing Date: 18-04-1997
(viii) Attorney/Agent Information:
(A) Name: Mirabel, Eric P.
(B) Registration Number: 31,211
(C) Reference/Docket Number: 97-1-PCT
(ix) Telecommunication Information:
(A) Telephone: (713) 664-2288
(B) Telefax: (713) 664-8914
(2) Information for SEQ ID NO:1:
(i) Sequence Characteristics:
(A) Length: 419
(B) Type: amino acid
(C) Strandedness:
(D) Topology: linear
(ii) Molecule Type
(A) Description: Sequence of the human IL-5 receptor protein
(xi) Sequence Description: SEQ ID NO:1:

Met Ile Ile Val Ala His Val Leu Leu Ile Leu Leu Gly

Ala Thr Glu Ile Leu Gln Ala Asp Leu Leu Pro Asp
15 20 25

595 Glu Lys Ile Ser Leu Leu Pro Pro Val Asn Phe Thr
30 35

Ile Lys Val Thr Gly Leu Ala Gln Val Leu Leu Gln
40 45

Trp Lys Pro Asn Pro Asp Gln Glu Gln Arg Asn Val
600 50 55 60

Asn Leu Glu Tyr Gln Val Lys Ile Asn Ala Pro Lys
65 70

Glu Asp Asp Tyr Glu Thr Arg Ile Thr Glu Ser Lys
75 80 85

605 Cys Val Thr Ile Leu His Lys Gly Phe Ser Ala Ser
90 95

Val Arg Thr Ile Leu Gln Asn Asp His Ser Leu Leu
100 105

Ala Ser Ser Trp Ala Ser Ala Glu Leu His Ala Pro
610 110 115 120

Pro Gly Ser Pro Gly Thr Ser Val Val Asn Leu Thr
125 130

Cys Thr Thr Asn Thr Thr Glu Asp Asn Tyr Ser Arg
135 140 145

615 Leu Arg Ser Tyr Gln Val Ser Leu His Cys Thr Trp
150 155

Leu Val Gly Thr Asp Ala Pro Glu Asp Thr Gln Tyr
160 165

Phe Leu Tyr Tyr Arg Tyr Gly Ser Trp Thr Glu Glu
620 170 175 180

Cys Gln Glu Tyr Ser Lys Asp Thr Leu Gly Arg Asn
185 190

Ile Ala Cys Trp Phe Pro Arg Thr Phe Ile Leu Ser
195 200 205

Lys Gly Arg Asp Trp Leu Ala Val Leu Val Asn Gly
210 215

Ser Ser Lys His Ser Ala Ile Arg Pro Phe Asp Gln
220 225

Leu Phe Ala Leu His Ala Ile Asp Gln Ile Asn Pro
230 235 240

Pro Leu Asn Val Thr Ala Glu Ile Glu Gly Thr Arg
245 250

Leu Ser Ile Gln Trp Glu Lys Pro Val Ser Ala Phe
255 260

Pro Ile His Cys Phe Asp Tyr Glu Val Lys Ile His
265 270 275

Asn Thr Arg Asn Gly Tyr Leu Gln Ile Glu Lys Leu
280 285

Met Thr Asn Ala Phe Ile Ser Ile Ile Asp Asp Leu
290 295 300

Ser Lys Tyr Asp Val Gln Val Arg Ala Ala Val Ser
305 310

Ser Met Cys Arg Glu Ala Gly Leu Trp Ser Glu Trp
315 320

Ser Gln Pro Ile Tyr Val Gly Asn Asp Glu His Lys
325 330 335

Pro Leu Arg Glu Trp Phe Val Ile Val Ile Met Ala
340 345

Thr Ile Cys Phe Ile Leu Leu Ile Leu Ser Leu Ile
350 355 360

Cys Lys Ile Cys His Leu Trp Ile Leu Leu Phe

Pro Ile Pro Ala Pro Lys Ser Asn Ile Lys Asp Leu
375 380

Phe Val Thr Thr Asn Tyr Glu Lys Ala Gly Ser Ser
385 390 395

Glu Thr Glu Ile Glu Val Ile Cys Tyr Ile Glu Lys Pro
400 405

Gly Val Glu Thr Leu Glu Asp Ser Val Phe
410 415

(2) Information for SEQ ID NO:2:
(i) Sequence Characteristics:
(A) Length: 16 amino acids
(B) Type: amino acid
(C) Strandedness:
(D) Topology: linear
(ii) Molecule Type
(A) Description: Sequence of the portion of human IL-5 receptor protein bound by antibody 165-4
(xi) Sequence Description: SEQ ID NO:2:

Ser Ile Gln Trp Glu Lys Pro Val Ser Ala Phe Pro Ile His Cys Phe
5 10 15

(2) Information for SEQ ID NO:3:
(i) Sequence Characteristics:
(A) Length: 27
(B) Type: nucleotide
(C) Strandedness: single
(D) Topology: linear
(ii) Molecule Type
(A) Description: primer
(xi) Sequence Description: SEQ ID NO:3:

TAAAGCTTGA CAGGATATGA TCATCGT 27

(2) Information for SEQ ID NO:4:
(i) Sequence Characteristics:
(A) Length: 22
(B) Type: nucleotide
(C) Strandedness: single
(D) Topology: linear

(ii) Molecule Type
(A) Description: primer
(xi) Sequence Description: SEQ ID NO:4:

700 TCGGATCCCA CATAAATAGG TT 22

WHAT IS CLAIMED IS:

1. Antagonist molecules which bind to the extracellular portion of the α chain of the IL-5 receptor protein, and inhibit proliferation of eosinophils as measured in an *in vitro* assay to the same or a greater degree than do the antibodies designated 165-13 and 165-4.
2. Antagonist molecules which bind to the same epitopes as those bound by the antibodies designated 165-13 and 165-4.
3. Antagonist molecules which bind to a conformational epitope and inhibit proliferation of eosinophils as measured in an *in vitro* assay to the same or a greater degree than does the antibody designated 165-13.
4. The antagonist molecules of any of claims 1 to 3 which are monoclonal antibodies or homologues, analogues, or fragments thereof.
5. The antagonist molecules of claim 4 which are chimeric, humanized or human monoclonal antibodies.
6. The antagonist molecules of claim 4 which are V_H, V_L, F_V, Fd, Fab or $(FAB')_2$ fragments of the murine, chimeric, humanized or human monoclonal antibodies.
7. Monoclonal antibodies having the same complementarity determining regions as the antibodies designated 165-13 or 165-4, or homologues, analogues or fragments thereof.
8. An antibody heavy chain having the same sequence as the antibodies

designated 165-13 or 165-4.

9. An antibody light chain having the same sequence as the antibodies designated 165-13 or 165-4.

10. An oligneucleotide encoding the antibody light or heavy chain of the antibody of claim 7.

11. An oligneucleotide encoding the sequence of the heavy or light chains of claims 9 or 10, respectively.

12. A host cell transfected with the oligneucleotide of claim 10.

13. A host cell transfected with the oligneucleotide of claim 11.

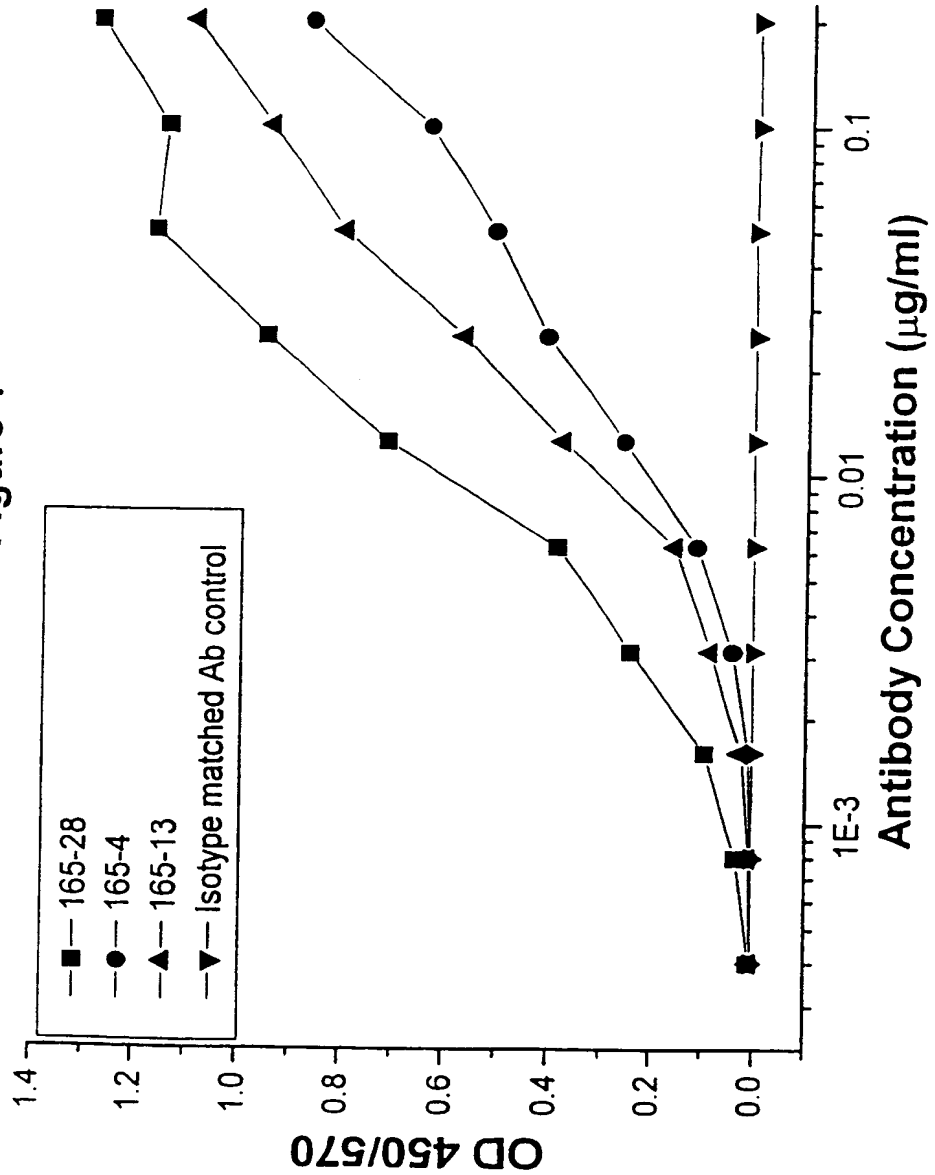
14. A process of making an antagonist to human IL-5 receptor, comprising:

(a) isolating molecules which bind to the human IL-5 receptor alpha chain;

(b) isolating the molecules from step (a) which compete with IL-5 for binding to the receptor protein.

15. The process of claim 14, further comprising the step of isolating the molecules which inhibit eosinophil proliferation *in vivo*.

Figure 1



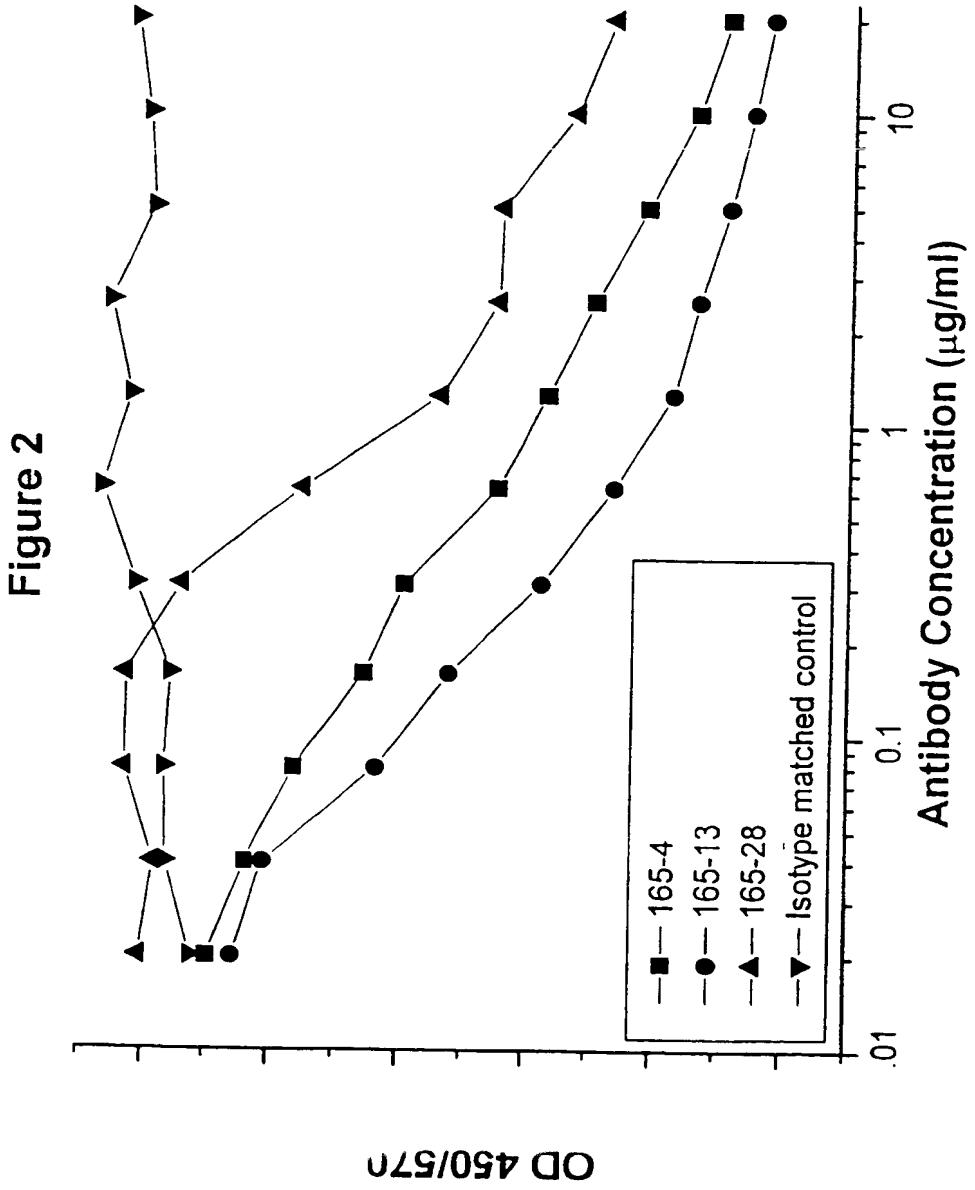

OD 450/570
.01
0.1
1
10
Antibody Concentration (μg/ml)
165-4
165-13
165-28
Isotype matched control

Figure 2

Figure 3

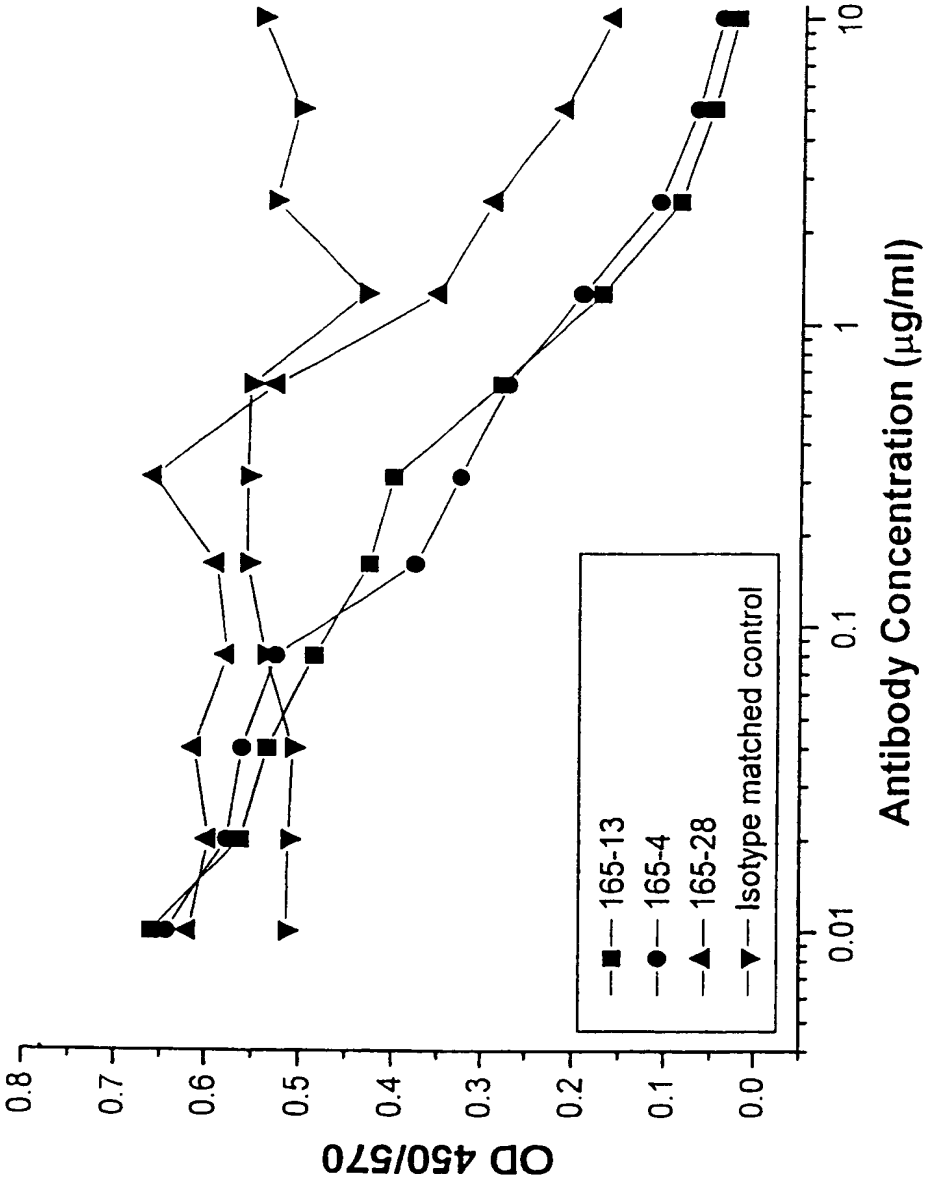
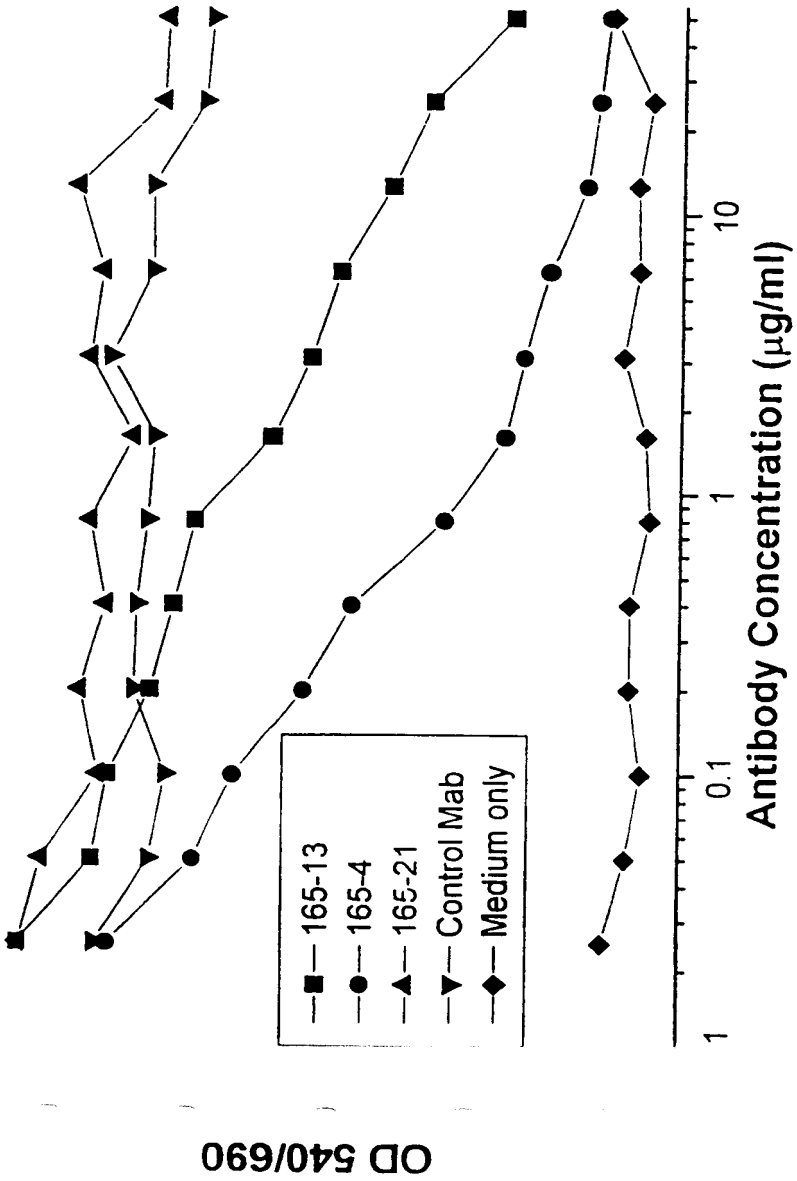


Figure 4 (A)


OD 540/690
Antibody Concentration (µg/ml)
1
0.1
1
10
165-13
165-4
165-21
Control Mab
Medium only

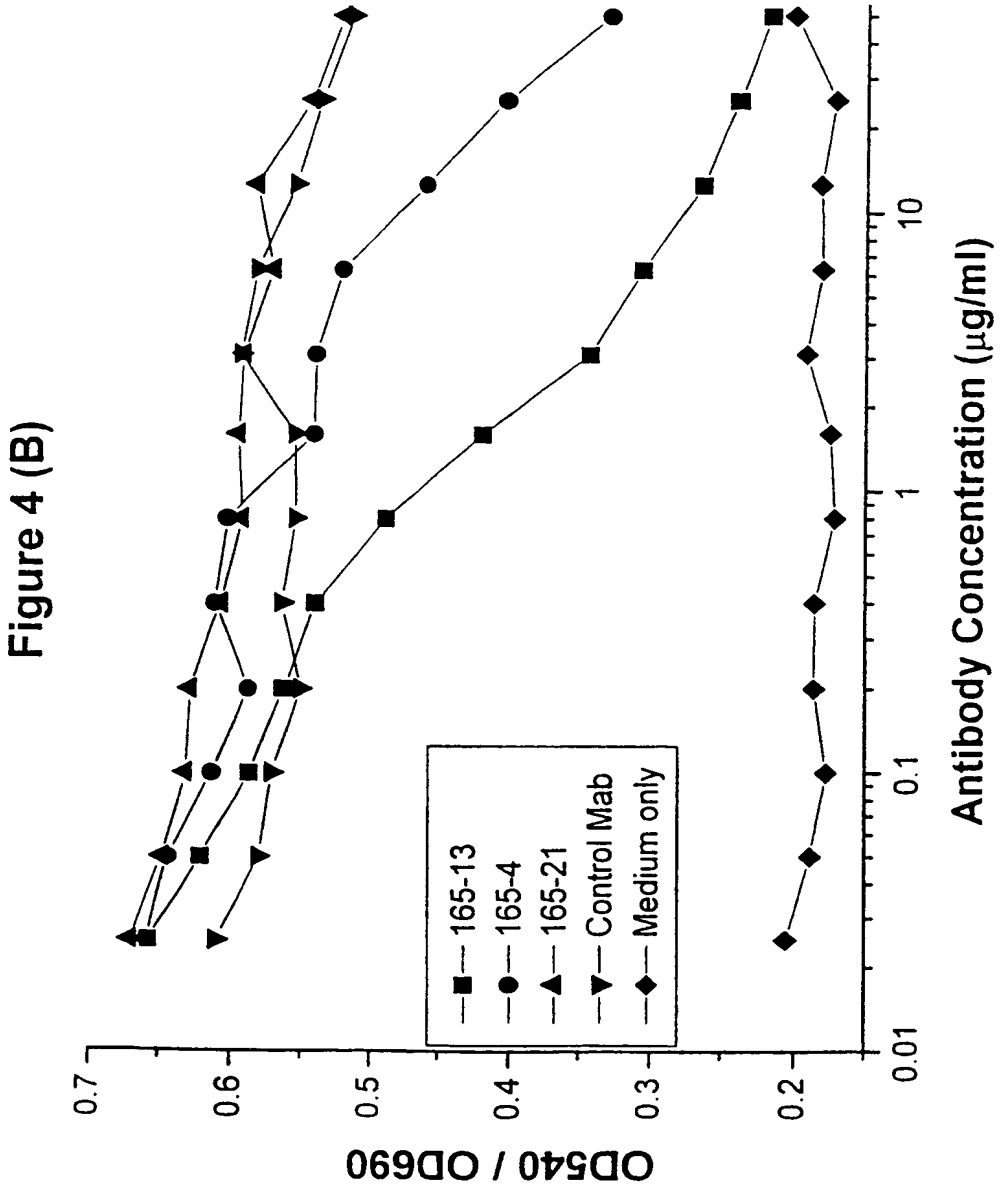
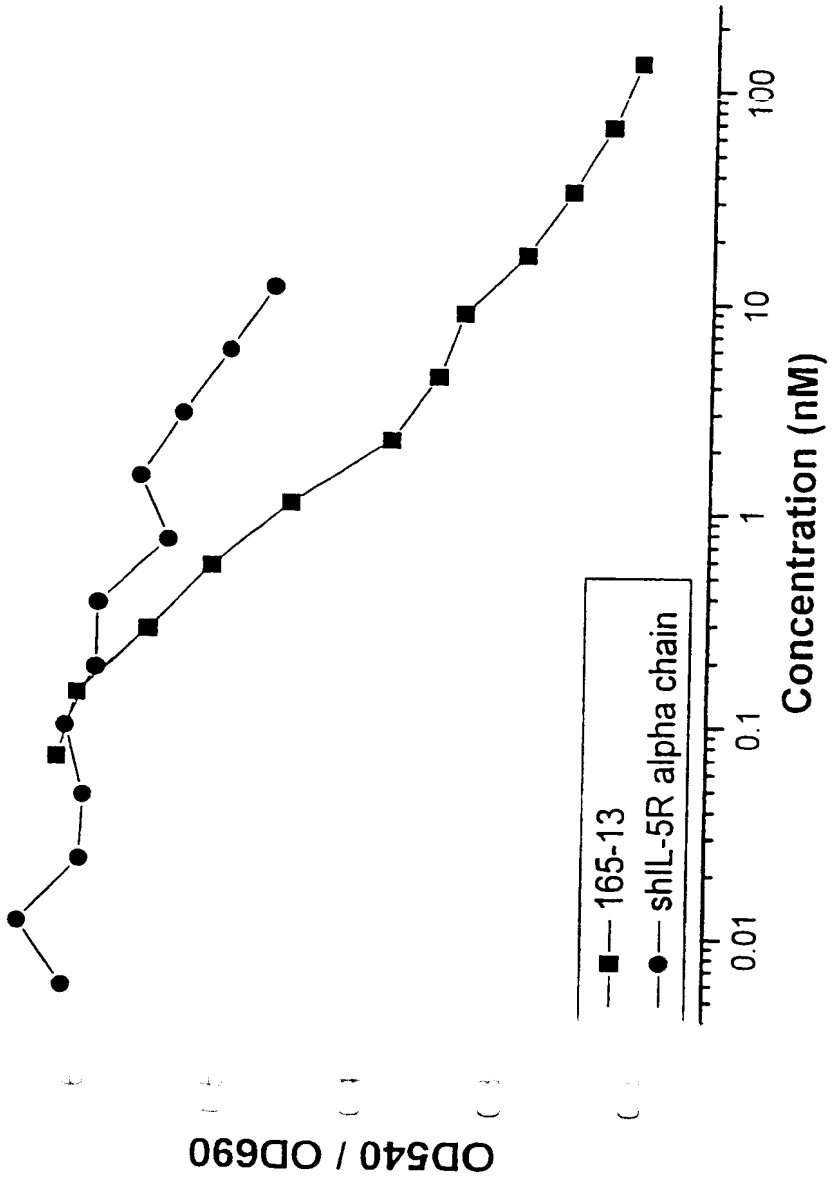


Figure 4 (B)

Figure 5


OD540 / OD690
Concentration (nM)
0.01
0.1
1
10
100
165-13
shIL-5R alpha chain

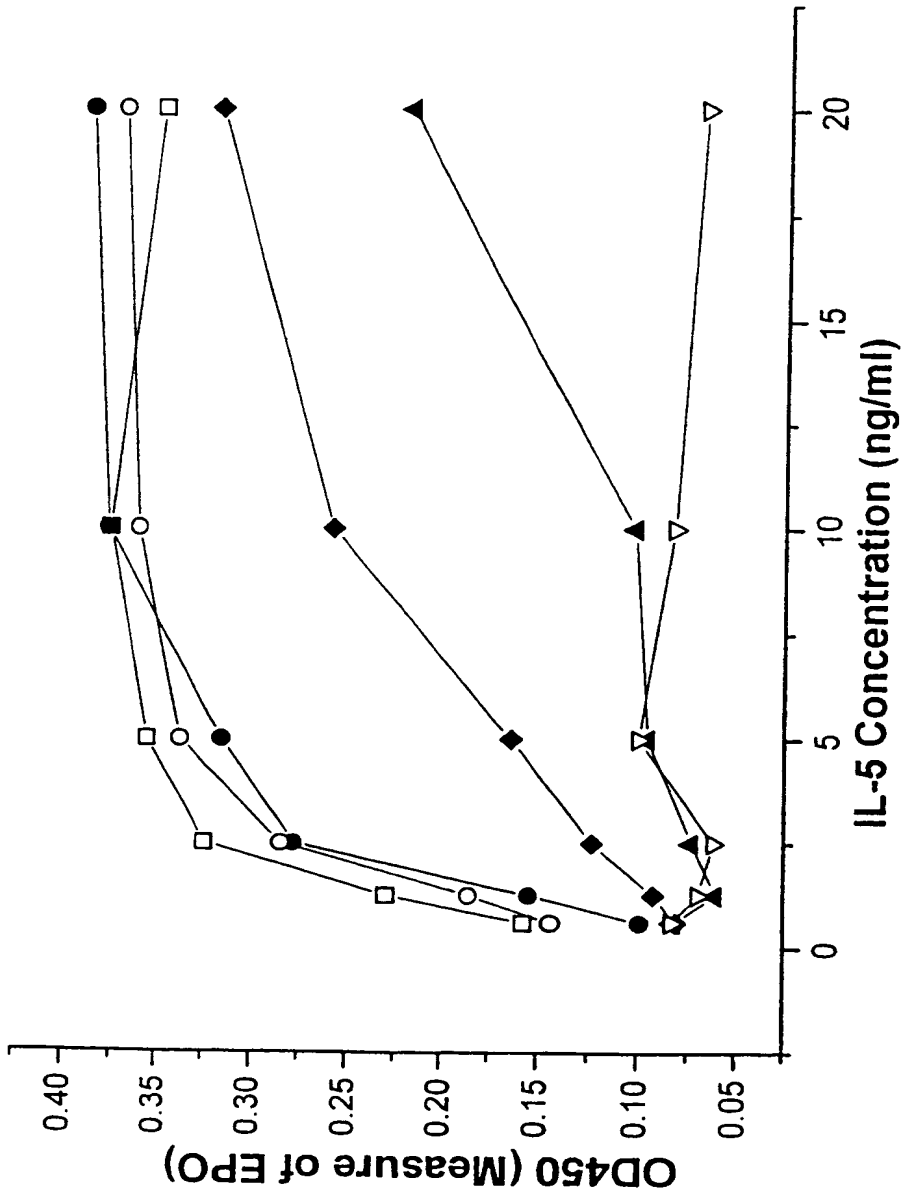


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07810

A CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 16/28; C07H 21/04; C12N 5/10; C12P 21/08; A61K 39/395
US CL : 530/388.22; 536/23.53; 435/325, 334, 70.1; 424/144.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.22; 536/23.53; 435/325, 334, 70.1; 424/144.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG biotech cluster
terms: interleukin 5, receptor, alpha, antibod?, eosinophil?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,455,337 A (DEVOS et al) 03 October 1995, especially examples 1 and 3.	1-8, 10-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search

03 JUNE 1998

Date of mailing of the international search report

17 JUL 1998

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